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GRANT NUMBER: DAMD17-94-J-4483

TITLE: Role of SHPTP2 in Mitogenic Signaling

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REPORT DATE: October 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
                U.S. Army Medical Research and Materiel Command
                Fort Detrick, Frederick, Maryland   21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
                          distribution unlimited

DTIC QUALITY INSPECTED 2

19961022 023

REPORT DOCUMENTATION PAGE

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE October 1996	3. REPORT TYPE AND DATES COVERED Annual (30 Sep 95 - 29 Sep 96)

4. TITLE AND SUBTITLE
Role of SHPTP2 Mitogenic Signaling

5. FUNDING NUMBERS
DAMD17-94-J-4483

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Our initial objectives were to determine the role of SHPTP2 in the growth factor (insulin and epidermal growth factor) activation of Ras. During the past funding period, these studies have lead us to identify a novel pathway directly involved in the down-regulation or inactivation of Ras following growth factor stimulation. We have been able to demonstrate that the inactivation of Ras occurs due to either an uncoupling of SOS from Grb2 (via insulin) or an uncoupling of Shc from Grb2-SOS (via EGF) due to a feedback serine/threonine phosphorylation of SOS. We have also recently demonstrated that these events occurs by a MEK-dependent but ERK-independent kinase. In order to further examine this critical regulatory pathway in the control of Ras activation/ inactivation, we will identify the functional insulin and EGF stimulated SOS phosphorylation sites by deletion analysis and by site-directed mutagenesis. In parallel, we will perform FPLC analysis to characterize and hopefully clone the specific kinase responsible for the functional phosphorylation of SOS. Finally, we will determine whether this phosphorylation is sufficient to account for the inactivation of Ras by performing in vitro dissociation studies and by expressing dominant-interfering mutants of the isolated SOS kinase.

14. SUBJECT TERMS
Breast Cancer
Mitogenesis, Ras, Phosphorylation, Photophase

15. NUMBER OF PAGES
15

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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_____   _____7/26/96_____
Principal Investigator's Signature   /Date

Grant No. DAMD17-94-J-4483

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A) INTRODUCTION:

Ras is a small GTP binding protein which provides for the integration of various extracellular cellular signals regulating mitogenesis, differentiation and development. The importance of Ras function is underscored by the observations that Ras is a potent oncogene and mutations which result in the persistent activation of Ras are associated with approximately 15% of all human tumors (1). Recently, substantial progress has been made in defining the molecular mechanisms by which tyrosine kinase receptors activate Ras-dependent signaling events. For example, insulin stimulation of the insulin receptor kinase results in the tyrosine phosphorylation of several substrates including the 185 kDa insulin receptor substrates 1 and 2 (IRS1/2) and isoforms of the src homology 2 (SH2) domain-containing $\alpha2$ collagen-related proteins, termed Shc (2-5). Tyrosine phosphorylation of IRS1 on Y895 and Shc on Y317 generates specific docking sites for the SH2 domain of Grb2, a 23 kDa adapter protein containing a single SH2 domain flanked by two SH3 domains (6-9). Numerous studies have established an association between the Grb2 SH3 domains and the carboxyl terminal proline-rich domain of SOS, a 170 kDa guanylnucleotide exchange factor for Ras (6,10-14). Recently it has been demonstrated that the expression of plasma membrane targeted forms of SOS result in the constitutive activation of Ras (15,16). Based upon these data, it has been suggested that plasma membrane targeting of the Grb2-SOS complex, by a Grb2-mediated association with tyrosine phosphorylated Shc and/or IRS1, accounts for the increased conversion of Ras from the inactive GDP-bound state to the active GTP-bound form (17,18). Alternatively, several studies have suggested that the carboxyl terminal domain of SOS functions as an auto-inhibitory domain which may be derepressed by the binding of Grb2 (19-21). In either case, the interaction of SOS with Grb2 plays an important role in regulating the activation state of Ras.

Once in the GTP-bound state, Ras associates with and activates members of the Raf family of serine/threonine kinases (22-26). In turn, activated Raf phosphorylates and activates the dual functional protein kinase MEK which then phosphorylates the ERK family of MAP kinases on both threonine and tyrosine residues (13,27). These phosphorylation events activate ERK and are required for the phosphorylation-dependent regulation of various cytosolic proteins and nuclear DNA binding transcription factors (28-30). Thus, the mitogenic actions of growth factors can be directly linked to transcriptional regulatory events utilizing Ras as a molecular switch converting upstream tyrosine kinase signals into a serine/threonine kinase cascade.

Although these data have provided a mechanism accounting for the activation and positive signaling role of Ras, Ras activation is transient and rapidly returns to its basal GDP-bound state (31-33). We and others have observed that insulin activation of the Ras/Raf/MEK/ERK pathway results in the serine/threonine phosphorylation of SOS, followed by dissociation of the Grb2-SOS complex (34-36). Furthermore, blockade of ERK activation by either expression of a dominant-interfering MEK mutant or with a specific MEK inhibitor prevents SOS phosphorylation, dissociation of the Grb2-SOS complex and prolongs the time Ras remains

GTP-bound (37,38). These data suggest that an insulin-stimulated feedback uncoupling of Grb2 from SOS may contribute to Ras desensitization.

In contrast to insulin, we have also observed that EGF induces a rapid transient activation of Ras. In addition, EGF induces the phosphorylation of SOS. However, EGF stimulation does not affect the interaction betwen the Grb2-SOS complex but instead induces a dissociation of the Grb2-SOS complex from tyrosine phosphorylated Shc (39). This difference in association state apparently resulted from differential targeting of these complexes by these growth factors. For example, insulin stimulation did not induce the persistent membrane targeting of Grb2 to the plasma membrane location of Ras whereas EGF stimulation resulted in approximately 50% of the cellular Grb2 pool found in a particulate fraction (39). In addition, expression of EGF receptor autophosphorylation mutants which are incapable of directing the association of SH2 domains did induce the dissociation of the Grb2-SOS complex in direct contrast to the wild type EGF receptor. Consistent with these data, mutants of Grb2 that can not associate with either Shc or the EGF receptor were fully capable of dissociating from SOS in both an insulin and EGF stimulated manner. Furthermore, membrane targeted forms of Grb2 prevented insulin from inducing a dissociation of the Grb2-SOS complex. Taken together, these data suggest that the plasma membrane targeting of the Grb2-SOS complex, in combination with serine/threonine phosphorylation has marked effects on its ability to remain associated and/or interact with Shc. Thus, although these growth factors both utilize the Shc, Grb2, SOS pathway to regulate the Ras activation/inactivation cycle they apparently do so by different molecular mechanisms.

Based upon these data, during the next time period we propose two related experimental approaches to determine the molecular basis for this difference in regulation of the Ras activation/inactivation cycle. In these experiments we will determine the sites of SOS phosphorylation by both insulin and EGF stimulation and examine both in vivo and in vitro the effects on Shc-Grb2-SOS interactions. In parallel, we will use FPLC analysis to identify the specific insulin and EGF stimulated kinase(s) that are responsible for the functional SOS phosphorylation in terms of its interaction with Grb2 and Shc.

B) BODY:

1. Experimental Procedures:

Cell culture - Chinese hamster ovary cells expressing both the human insulin and EGF receptors (CHO/IR/ER) were prepared as previously described by co-transfection of CHO/IR cells with a hygromycin resistant plasmid (pRBK, Invitrogen) and the human EGF expression plasmid (40). These cells were maintained in alpha-minimal Eagle's medium supplemented with nucleosides, 200 µg/ml hygromycin, 500 µg/ml neomycin and 10% fetal bovine serum. NIH-3T3 fibroblasts were engineered to express the human wild type EGF receptor (ER-WT) and an EGF receptor mutant (ER-5F) in which the five major tyrosine autophosphorylation sites (Y992, Y1068, Y1086, Y1148, Y1173) were changed to phenylalanine as previously

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described (41). These cells were grown in Dulbecco's modified Eagle's medium supplemented with nucleosides, 500 µg/ml neomycin and 5% calf serum.

Grb2 cDNA expression constructs - The wild type myc-tagged human Grb2 gene was cloned into the mammalian expression vector, CLDN (obtained from SmithKline Beecham). The PstI/BglII and BglII/XbaI fragments from pBSKGrb2mycAAA (42) were subcloned into the PstI/XbaI sites of the pALTER-1 vector (Promega) to create pALTER-Grb2mycΔ5'. The PstI fragment from pBSKGrb2mycAAA was inserted into the PstI site of pALTER-Grb2mycΔ5' to create pALTER-Grb2myc (correct orientation was verified by restriction analysis). The BamHI fragment of pALTER-Grb2myc (contains the full Grb2myc sequences) was then subcloned into CLDN at the BamHI site of the multi-cloning site to create CLDN-Grb2myc. The cDNA clones were screened for orientation of the BamHI fragment, and sequences were verified by automated DNA sequencing at The University of Iowa DNA Core Facility. The cloning strategy for CLDN-Grb2mycR86K was identical with the exception that in the first cloning step, the PstI/BglII fragment for the SH2 mutant (R86K) was from the plasmid pGEX-2TK/Grb2 R86K (43). The wildtype Grb2myc sequences were also engineered as a gene fusion with the extracellular and transmembrane domain of the human A2 allele (44), and subcloned into the mammalian expression vector, pcDNA3 (Invitrogen), to create pcDNA3-A2/Grb2-myc.

Transient transfection by electroporation - We have previously demonstrated that electroporation can be used to express various cDNAs in CHO/IR with a 85-100% transfection efficiency (45). Briefly, CHO/IR/ER cells were electroporated with various plasmid DNAs (2 µg) at 340 volts and 960 µF. Thirty h following transfection the cells were serum starved for 3 h and either untreated or incubated for various times in the absence or presence of 100 nM insulin or 20 nM EGF.

Immunoblotting and immunoprecipitation - Whole cell detergent extracts were prepared by solubilization in lysis buffer (50 mM HEPES, pH 7.8, 1% Triton X-100, 2.5 mM EDTA, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium vanadate, 2 mM pepstatin, 0.5 trypsin inhibitory units of aprotinin, 1 mM phenylmethylsulfonylfluoride and 10 µM leupeptin). Immunoprecipitation and immunoblotting was performed using monoclonal ERK (Zymed), monoclonal Shc (Transduction Laboratories), monoclonal SOS (Transduction Laboratories), monoclonal EGF receptor (LA1, Upstate Biotechnology Inc.), monoclonal phosphotyrosine (PY20, Santa Cruz Biotechnology) and monoclonal Grb2 (Transduction Laboratories) antibodies as previously described (36,37). The Grb2-myc fusion proteins were immunoprecipitated with the 9E10 myc epitope monoclonal antibody. The immune complexes were resolved by SDS-PAGE and electrophoretically transfered to PVDF membranes (Millipore). The primary monoclonal and polyclonal antibodies (except PY20-HRP) were detected with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG, respectively (BioRad) and visualized by the enhanced chemiluminescence detection system (Amersham).

Cytosolic versus particulate fractionation - CHO/IR/ER cells expressing the various Grb2 fusion proteins were were separated into cytosolic (supernatant) and membrane (particulate) fractions as previously

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described by Aronheim et al. (15), with minor modifications. Transfected cells were either left untreated or stimulated with insulin or EGF for 5 min and resuspended in 500 µl of ice cold detergent-free buffer (50 mM HEPES, pH 7.8, 2.5 mM EDTA, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium vanadate, 2 mM pepstatin, 0.5 trypsin inhibitory units of aprotinin, 1 mM phenylmethylsulfonylfluoride and 10 µM leupeptin). The cell suspension was sonicated (Fisher Scientific 550 Sonic Dismembrator) for 3 x 10 s and was spun in a microfuge for 30 min at 4°C. The supernatant was directly transferred into Laemmli sample buffer. The pellet was washed with an additional 500 µl ice cold detergent-free buffer, resuspended in 500 µl Laemmli sample buffer and boiled for 10 min. Proteins (1/20 of each fraction) were separated on 10% SDS-polyacrylamide gels and Western blotted as described above using the 9E10 myc monoclonal antibody.

Precipitation of SOS with the GST-Grb2 fusion protein - The GST-Grb2 fusion protein bound to glutathione-Sepharose beads (25 µl bed volume) was prepared as previously described (36). The glutatione-Sepharose bound GST-Grb2 fusion protein was then incubated for 1 h at 4°C with whole cell lysates (50 mM Hepes, pH 7.8, 1% Triton X-100, 2.5 mM EDTA, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium vanadate, 1 mM phenylmethylsulfonylfluoride, 2 µM pepstatin, 0.5 trypsin inhibitory units of aprotinin and 10 µM leupeptin) isolated from either unstimulated, 100 nM insulin or 20 nM EGF-stimulated CHO/IR/ER cells for 20 min. The Sepharose beads were pelleted and washed three times with phosphate buffered saline and solubilized in Laemmli sample buffer. The amount of SOS associated with the immobilized GST-Grb2 fusion protein was determined by immunoblotting with a SOS antibody.

SOS in vitro kinase assay - Cells which had been transfected with MKP-1 or vector, and treated as indicated in the figures, were harvested in lysis buffer (50 mM HEPES, pH 7.8, 1% Triton X-100, 2.5 mM EDTA, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium vanadate, 2 mM pepstatin, 0.5 trypsin inhibitory units of aprotinin, 1 mM PMSF and 10 µM leupeptin). The extracts (30 µl) were incubated with 1 µg of epitope (Glu-Glu)-tagged hSOS1 bound to anti-Glu-Glu monoclonal antibodies conjugated to Protein G-Sepharose (46). The kinase reaction was initiated with the addition of 100 µl of kinase buffer (20 mM HEPES, pH 8.0, 20 mM MgCl$_2$, 20 mM β-glycerolphosphate, 0.1 mM sodium vanadate, 2 mM DTT, 40 µM ATP and 5 µCi [γ-^{32}P]ATP). The reactions were incubated for 30 min at room temperature with gentle agitation and terminated by addition of 1 ml of ice cold HEPES binding buffer. The beads were then pelleted and the proteins were heated at 100°C in Laemmli sample buffer for 5 min and resolved on a 7.5% SDS gel. The gels were stained with Coomassie Blue, destained, dried and subjected to autoradiography. In the case of the fractions from the MonoQ column, 30 µl from each 1 ml fraction were assayed identically as described above for SOS kinase activity.

MonoQ column chromatography - Cells which had been transfected with MKP-1 or empty vector, and treated as indicated in the figures, were harvested in lysis buffer (50 mM HEPES, pH 7.4, 1% Triton X-100, 2.5 mM EDTA, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium vanadate, 2 mM pepstatin, 0.5 trypsin inhibitory units of aprotinin, 1 mM PMSF and 10

µM leupeptin). The extracts were cleared and 1 mg of protein was made to 10 ml with buffer B (50 mM HEPES, pH 7.4, 2.5 mM EDTA, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium vanadate, 1 mM PMSF), and applied to a HR5/5 MonoQ Fast Protein Liquid Chromatography (FPLC) column. Chromatography was performed using a flow rate of 0.25 ml/min with a 20 ml linear gradient of 0 to 1 M NaCl in buffer B beginning at fraction 15. Fractions (1 ml) were collected and stored at -80°C.
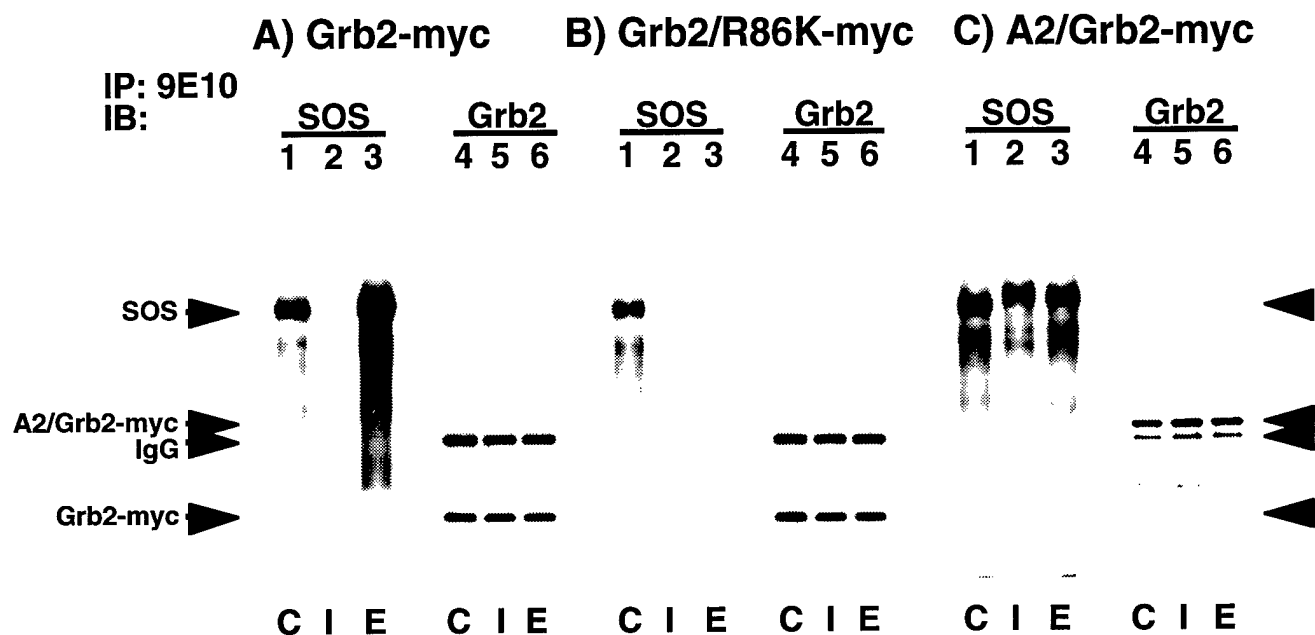
2. Results:

Previously, we were able to demonstrate that the protein tyrosine-specific phosphatase SHPTP2 functions as a positive upstream regulator of Ras activation and mitogeneis (47). We also identified an approximate 115 kDa species that was tyrosine phosphorylated in response to both insulin and EGF, but not PDGF, which was directly associated with SHPTP2 (48,49). Although we have currently been unsuccessful in either identifying and/or purifying this protein, during the course of these studies we observed that the Ras guanylnucleotide exchange factor SOS was serine/threonine phosphorylated in response to both insulin and EGF stimulation. Currently, we are continuing our attempts to purify this 115 kDa protein. However, these studies have provided additional important information also directly related to the regulation of Ras function. Thus, during the past funding period in parallel to our ongoing attempts to identify the 115 kDa SHPTP2 binding protein, we have made substantial progress in determining the regulatory role of SOS phosphorylation in the Ras activation/inactivation cycle.

We and others have previously observed that stimulation of numerous cell types with growth factors, serum or phorbol esters resulted in a serine/threonine phosphorylation of SOS. We had also observed that in the case of insulin, PDGF, serum and TPA this phosphorylation event correlated with a dissociation of the Grb2-SOS complex. Furthermore, the dissociation of the Grb2-SOS complex occurred in parallel with the inactivation of Ras from the GTP-bound back to the GDP-bound state. However, we recently found that although EGF stimulation also induced a serine/threonine phosphorylation of SOS, there was no effect on the association between Grb2 and SOS (39). To investigate the molecular basis for this difference, we had engineered a mammalian expression vector containing the wild type Grb2 cDNA fused to a myc epitope tag (Fig. 1).

Transfection of CHO/IR/ER cells with the Grb2-myc cDNA followed by immunoprecipitation with the myc antibody demonstrated the expected co-immunoprecipitation of the SOS protein (Fig. 1A, lane 1). As previously reported, insulin stimulation resulted in a marked decrease in the amount of SOS that could be co-immunoprecipitated with the Grb2-myc protein (Fig. 1A, lane 2). Surprisingly, however EGF stimulation did not decrease the amount of Grb2-myc associated with SOS and, in fact, typically resulted in an increase (Fig. 1A, lane 3). To demonstrate that the expression and immunoprecipitation of the Grb2-myc protein was identical under these conditions, a Grb2 immunoblot was performed on the myc immunoprecipitates (Fig. 1A, lanes 4-6). Based upon these and other data, we speculated that the difference between insulin and EGF signaling may be due to the ability of the EGF receptor to directly target SH2 domain containing proteins persistently to the plasma membrane. To address this

issue, we next expressed a Grb2 mutant (Grb2/R86K-myc) in which the essential arginine in the SH2 domain was replaced with lysine. In contrast to the wild type Grb2-myc protein, expression of Grb2/R86K-myc resulted in both an insulin and EGF-stimulated dissociation from SOS (Fig. 1B, lanes 1-3). These data further suggest that the difference in the insulin- and EGF-stimulated targeting of the Grb2-SOS complex through the Grb2 SH2 domain is an important event regulating the dissociation of the Grb2-SOS complex.

A) Grb2-myc   B) Grb2/R86K-myc   C) A2/Grb2-myc

IP: 9E10
IB:

SOS	Grb2	SOS	Grb2	SOS	Grb2
1 2 3	4 5 6	1 2 3	4 5 6	1 2 3	4 5 6

SOS

A2/Grb2-myc
IgG

Grb2-myc

C I E   C I E   C I E   C I E   C I E   C I E

The differences in Grb2-SOS targeting induced by insulin and EGF receptor activation could reflect either a direct physical association of the Grb2-SOS complex with the EGF receptor itself or via a general association of the complex with the plasma membrane. To distinguish between these possibilities, we prepared a plasma membrane-associated form of Grb2 by constructing a cDNA to encode a fusion protein containing the extracellular and transmembrane domains of the human A2 MHC class I protein in-frame with wild type Grb2-myc (44). Similar to the cytosolic forms of Grb2, the expressed membrane-bound form of Grb2 (A2/Grb2-myc) also associated with SOS in unstimulated cells (Fig. 1C, lane 1). However, neither insulin nor EGF induced dissociation of this Grb2 fusion protein from SOS (Fig. 1C, lanes 2 and 3). Thus, under these conditions the insulin regulation of Grb2-SOS interaction was essentially identical to that observed for EGF.
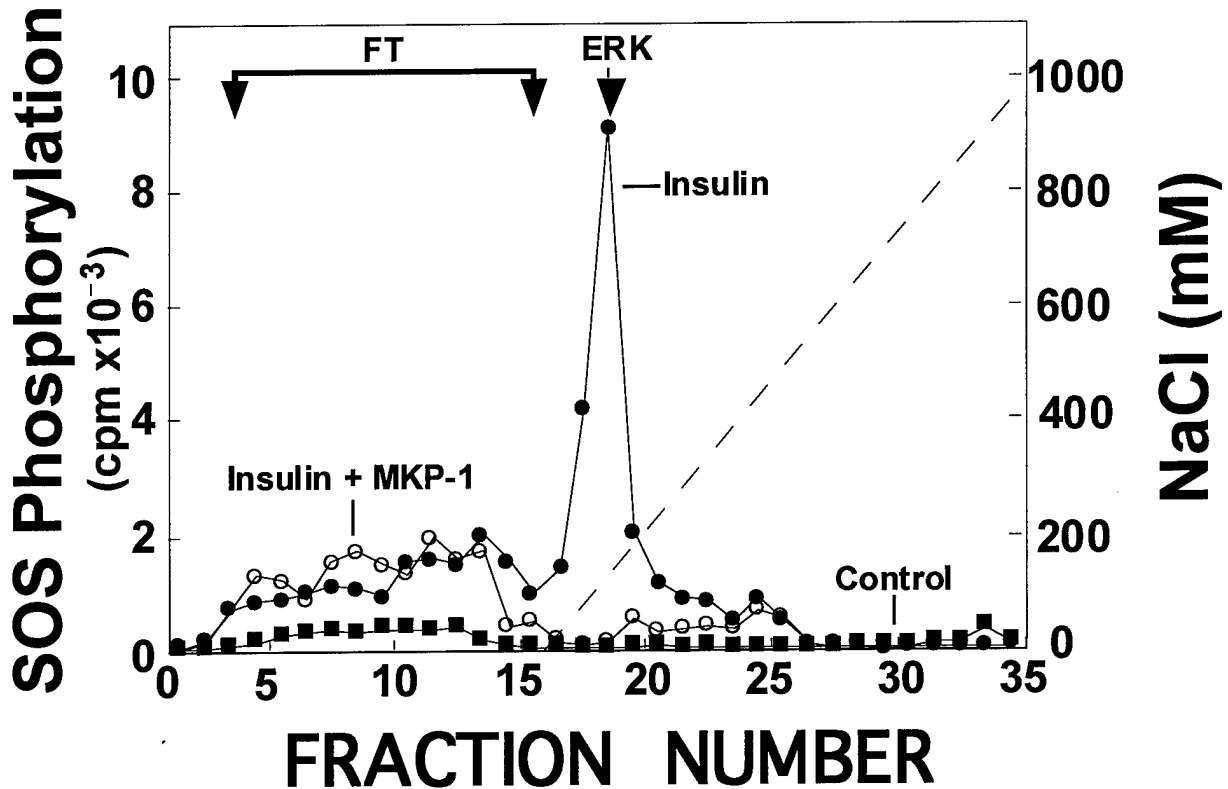
There are several possibilities to account for a role in membrane targeting in the regulation of the dynamic interaction between Grb2-SOS. One possibility is that the EGF induced membrane/receptor association influences the specific sites of SOS phosphorylation compared to other growth factors. However, the neither the kinases nor the sites of SOS phosphorylation have been identified. Thus, during the past year we have also established an *in vitro* kinase assay using baculovirus purified SOS as a substrate. Using this assay system as well as other in vivo analysis, we were able to demonstrate that SOS phosphorylation was MEK-dependent but ERK-independent. This was primarily based upon our ability to inhibit ERK activity by expression of the MAP kinase phosphatase, MKP-1 (46). We therefore designed experiments to further characterize the SOS phosphorylation activity. Whole cell extracts were applied to a Fast Phase Liquid Chromatography MonoQ column and the resultant fractions assayed for SOS phosphorylating activity (Fig. 2). Extracts from insulin-stimulated cells displayed two major peaks of SOS phosphorylating activity (solid circles). The sharp peak of activity eluting at approximately 200 mM NaCl (fractions 16-20) contains activated ERK1/2 proteins as detected by immunoblotting (data not shown). Consistent with previous studies (50), we did not detect the ERK proteins in any of the other fractions. The other region of SOS phosphorylating activity was a broad peak which did not bind to the MonoQ column but which was present in the flow through fractions (fractions 3-13). As expected, extracts from unstimulated cells had relatively low levels of SOS phosphorylating activity in all the column fractions (closed squares). Importantly, extracts from insulin-stimulated cells expressing MKP-1 also displayed

essentially no SOS phosphorylating activity in fractions 16-20 (open circles) which contained the inactive ERK1/2 proteins as detected by immunoblotting (data not shown). However, the SOS phosphorylating activity in the void volume was completely unaffected by the expression of MKP-1. These data further document that the hormone-stimulated SOS phosphorylating activity was physically distinct from the ERK proteins.

IB: Flag

	SOS/CT	S1099A T1102A T1105A
	1 2 3	4 5 6

SOS/CT
NS

C I E C I E
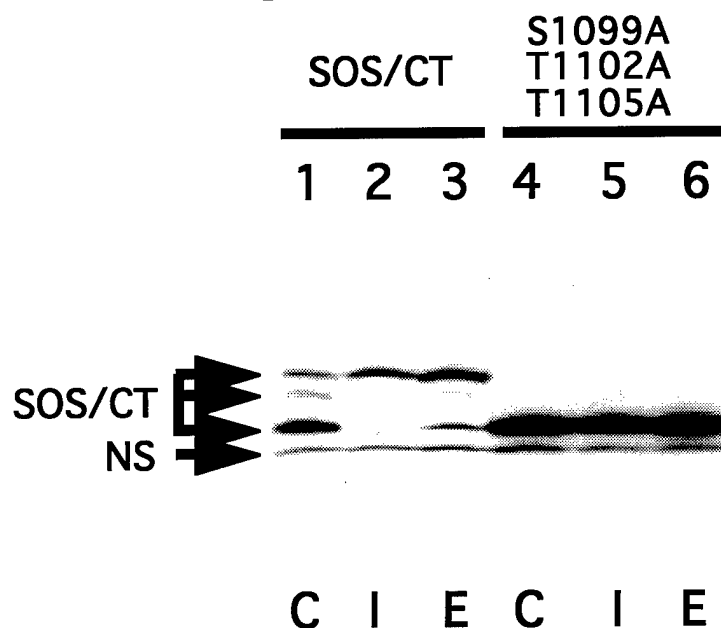
To identify the sites of SOS phosphorylation, we initially expressed a series of SOS deletions mutants containing the Flag epitope tag and determined which domain was required for the phosphorylation and dissociation of the Grb2-SOS complex. These data demonstrated that the carboxyl terminal proline-rich domain (residues 1072-1337) was sufficient for these functions. Based upon these data, we next generated a several site specific mutants within this carboxyl terminal domain. As presented in Figure 3, expression of the carboxyl terminal domain of SOS (SOS/CT) resulted in the presence of three specific protein bands corresponding to the unphosphorylated truncated SOS protein (~30 kDa) and two higher molecular species corresponding to partially and fully phosphorylated SOS (Fig. 3, lane 1). The Flag epitope immunoreactive band at ~25 kDa is a non-specific protein (NS) in whole cell lysates that cross reacts with this antibody. In any case, following insulin or EGF stimulation it is apparent that there is a marked decrease in electroporetic mobility due to the phosphorylation of SOS (Fig. 3, lanes 2 and 3). In contrast, expression of the triple mutant (S1099A, T1102A, T1105A) in which three potential serine/threonine phosphorylation sites were mutated to alanine completely prevent both the insulin and EGF stimulated phosphorylation (Fig 3., lanes 4-6). We are currently examining the single mutants as well as determining the interaction of these mutants with Grb2.

C) CONCLUSIONS:

At present, our efforts remain focused on determining the regulation of Ras function. When this projected originated, we had observed that the protein phosphatase SHPTP2 played a positive signaling role in Ras activation and that it associated with a 115 kDa tyrosine phosphorylated protein. During the past year, we have attempted to purify this protein by GST-SH2 affinity chromatography and phosphotyrosine affinity chromatography. Unfortunately, we have not yet been successful and have recently purchased a FPLC system to aid us in our further attempts to purify and identify this protein. However, in our studies characterizing this protein in tissue culture cell lines, we observed that the Ras guanylnucleotide exchange factor SOS was serine/threonine phosphorylated which apparently modulates its affinity for Grb2. Thus, in parallel we have also pursued this issue and have made progress in characterizing both the kinase and specific phosphorylation sites that are responsible for the dissociation of SOS from Grb2.

In the next year, we plan to continue our efforts to purifiy, sequence and clone the 115 kDa SHPTP2 binding protein. This will be approached in two independent manners. Since we have been unsuccessful in using GST-SH2 and phosphptyrosine antibody affinity chromatography, we will FPLC column chromatography instead. First, unstimulated and growth factor stimulated cell extracts will be applied to a MonoQ column as described above. The fractions will then either be directly subjected to phosphotyrosine immunoblotting or immunoprecipitated with the SHPTP2 antibody followed by phosphotyrosine immunoblotting. If the MonoQ column does not yield sufficient purity for antibody production and/or microsequencing, we will then apply the MonoQ partially purified material on a MonoS column. We anticipate that a combination of MonoQ and MonoS affinity chromatography will provide sufficient purification to identify this protein. In addition, as this progress report was being prepared two groups apparently cloned the drosophila homolog of the 115 kDa SHPTP2 binding protein, DOS for Daughter of Sevenless directly implicated in Ras activation (51,52). Based upon these data, we are currently designing a series of PCR primers that will be used to amplify the mammalian counterpart. Alternatively, we may also use low stringency hybridization of a mammalian cDNA library using the drosophila DOS cDNA as a probe.

Since our initial studies have also demonstrated a critical role of SOS in the regulation of Ras we also plan to continue these efforts. Thus, we also plan to purifying the kinase responsible for SOS phosphorylation and more precisely determine the specific sites of SOS phosphorylation responsible for mediating its interaction with Grb2. Although these studies will be performed in parallel, it is important to recognize that both approaches are directed at elucidating the complex regulation of the Ras activation/inactivation cycle which plays a central role in the control of mitogenesis and cellular transformation.

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